

Role of Lipopolysaccharide in Colonization of the Mouse Intestine by *Salmonella typhimurium* Studied by In Situ Hybridization

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An avirulent, streptomycin-resistant *Salmonella typhimurium* strain, SL5319, and its lipopolysaccharide (LPS)-deficient mutant strain, SL5325, differ in their ability to colonize the large intestines of streptomycin-treated mice. When fed to mice independently, the strains colonize equally well, but when fed together, the LPS-deficient mutant is outcompeted by the wild-type strain during establishment in the gut (J. J. Nevola, B. A. D. Stocker, D. C. Laux, and P. S. Cohen, *Infect. Immun.* 50:152–159, 1985). In the present study, the spatial distribution in the intestinal mucosal layer of the two strains was visualized by specific hybridization to bacterial rRNA in histological sections of mouse colon and cecum. The first day after infection, 9.8% of the smooth SL5319 cells observed in mucus were found to be associated with the mouse epithelial cells, but three days after infection, the corresponding fraction of adhering bacteria was reduced to 2.1%. The LPS-deficient *S. typhimurium* strain was confined to the part of the mucosal layer closest to the colonic lumen and was not observed to adhere to the epithelium either at day 1 or 3 after infection. Quantitative determinations of the distance from the *S. typhimurium* cells to the epithelial wall confirmed that the average distance for the rough *S. typhimurium* SL5325 was much larger than for its smooth counterpart, *S. typhimurium* SL5319. Quantification of the hybridization signal from bacteria isolated from the cecal mucus revealed that the two strains had the same ribosome concentration, indicating that they have the same potential for growth in the intestinal environment. On the basis of these observations, we suggest that the better colonization ability of the strain carrying wild-type LPS is due to the better abilities to penetrate the intestinal mucosal layer and to subsequently bind to the epithelial cells in vivo.

Bacterial membrane-associated lipopolysaccharide (LPS) plays a major role in host-pathogen interaction and is generally accepted as an important antigen (11). LPS of the enteric pathogen *Salmonella typhimurium* has been shown to induce serum immunity in mice (1, 8). The importance of LPS in colonization and interaction with intestinal mucosa has also been recognized. For *Escherichia coli*, LPS appears to be a major bacterial adhesin for specific binding to one or more of the glycoprotein receptors present in mouse colonic mucus (7). Furthermore, it was observed that LPS is necessary for *Klebsiella pneumoniae* to colonize the gut of the germfree chicken (5).

The role of LPS in the ability of *S. typhimurium* to colonize the large intestines of streptomycin-treated mice has been the object of a number of studies (14, 17, 18). It was reported that the avirulent, streptomycin-resistant *S. typhimurium* SL5316 strain containing wild-type LPS is a better colonizer of the intestines of streptomycin-treated mice than LPS-deficient mutants of this strain. When fed to mice alone, the LPS-deficient mutant strains colonized the gut, but when introduced simultaneously with an *S. typhimurium* strain carrying wild-type LPS, the mutants were rapidly outcompeted (18). It was further-

more reported that the reduced colonization ability of LPS-deficient mutants was not caused by alterations in motility and chemotactic response (14). Because of the observations that the wild-type and mutant strains had similar growth rates in mucus in vitro and that LPS-deficient strains in vitro travelled through a layer of mucus far slower than the parental strain with wild-type LPS, it was suggested that the reduced colonization ability of the mutants was caused by a reduced ability to penetrate the intestinal mucus (17).

Hybridization of whole bacterial cells with fluorophore-labelled, specific oligonucleotide probes targeting the rRNA has proven to be a powerful tool to visualize the spatial distribution of specific bacterial species in the intestinal mucosal layer (21). Moreover, ribosomal hybridization allows relative quantification of the concentration of ribosomes in the bacteria and thereby allows the assessment of recent or present growth rates in situ (20, 22). Therefore, this method can be used to directly test whether the explanations for the different colonization abilities suggested by Nevola et al. (17) and McCormick et al. (14) are valid in situ. In the present study, we used the hybridization method to study the colonization characteristics of the avirulent *S. typhimurium* SL5319 strain, which is smooth, and SL5325, which carries an *rfaJ* mutation and consequently has a modified LPS lacking the O antigen (18). The data presented concern two aspects of the colonization process in the mouse gut: (i) apparent growth rates of bacteria in intestinal mucus measured by in situ rRNA hybridization to the two strains, and (ii) the locations of the two strains of *S. typhimurium* relative to the epithelium and the mucosal layer of the large intestine.

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MATERIALS AND METHODS

Bacterial strains. Nonvirulent *S. typhimurium* strains were needed in order to be able to monitor colonization of the mucosal layer for a longer period without killing the mouse. The strains used in this study originate from the streptomycin-resistant *S. typhimurium* SL5316 strain, which is avirulent because of an *aroA* mutation (18). Strain SL5319 is SL5316 made *hisD857::Tn10* by transduction. Strain SL5325 is a spontaneous nalidixic acid-resistant, *rfaJ989* mutant of SL5316, consequently lacking the O side chain, i.e., the *N*-acetylglucosamine and the glucose II unit of the LPS (17, 18).

Growth media. Platings were made on L-broth agar plates (Statens Serum-institut, Copenhagen, Denmark). Liquid cultures were grown either in L broth or in AB minimal medium (6) supplemented with 50 μ g (each) of L-histidine, L-leucine, L-phenylalanine, L-tryptophan, and L-tyrosine per ml and 1 μ g (each) of *p*-aminobenzoic acid, *p*-hydroxybenzoic acid, and 2,3-dihydroxybenzoic acid per ml. Bacteria were grown in the presence of 100 μ g of streptomycin sulfate per ml. Amino acids, benzoic acids, and streptomycin sulfate were purchased from Sigma Chemical Co., St. Louis, Mo.

Measurements of bacterial growth in defined media. Growth rates of pure cultures were measured by monitoring the optical density at 450 nm. Generation times are expressed in minutes or as specific growth rates, i.e., $\ln 2$ /doubling time (hours^{-1}).

Determination of RNA/protein ratios. RNA contents in bacteria were determined by the method of Schneider (23), using a specific color reaction with Orcinol (Sigma). Protein contents were determined by the use of the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Fixation of whole bacterial cells. Cells were fixed in 3% paraformaldehyde as previously described (2, 21). Fixed cells were stored at -20°C until use.

Preparation of mucus for in vitro incubation. Two mice were treated for 24 h with 5 g of streptomycin sulfate per liter of drinking water. The mice were then euthanized, the ceca were removed and nicked, and cecal contents were carefully scraped out. The ceca were washed in 5 ml of cold 0.9% (wt/vol) sodium chloride after which the mucus was scraped from the intestinal wall with a rubber spatula. The mucus was kept at -20°C until use.

In vitro incubation in mucus. *S. typhimurium* strains were grown in supplemented AB minimal medium (described above) with 0.4% (wt/vol) glucose and 1.0% (wt/vol) Casamino Acids up to a density of about 10^8 bacteria per ml as estimated by spectrophotometry. Samples of 1 ml were taken, and cells were fixed as described above. At the same time, other 1-ml samples were concentrated 100-fold and diluted with nonsterilized mucus from the ceca of two streptomycin-treated mice to a final density of about 10^{10} bacteria per g of mucus. The bacteria were incubated for 1 h at 37°C in mucus, whereafter smears were prepared, fixed, and hybridized as described below for bacterial smears.

Mouse colonization experiments. Five- to eight-week-old outbred albino female SS:CF1 mice (Statens Seruminstitut) were given sterile water containing 5 g of streptomycin sulfate per liter. After 24 h, 2.2×10^8 (for growth rate studies) or 3.0×10^{10} (for location studies) *S. typhimurium* cells in 100 μ l of 20% (wt/vol) sucrose was given per os to the mice. During the colonization experiments, the mice were individually caged, the cages were changed daily, and the mice continuously received drinking water containing streptomycin sulfate (5 g/liter). For preparation of histological sections, two mice were euthanized on days 1, 3, and 6 after infection. For preparation of bacteria for quantification of ribosomes, two mice were euthanized on day 6 after infection. In all cases, one uninfected, streptomycin-treated mouse was used as a control. All experiments were performed at least twice.

Preparation of bacterial cell smears. To prepare bacterial cell smears for hybridization, the method previously described (22) was slightly modified. The cecum was removed from the euthanized mouse and placed in a weighed, sterile petri dish. After the cecum was nicked, it was cleaned, and the remaining luminal contents were transferred to a new sterile, weighed petri dish. The mucosal layer was carefully scraped off with a rubber spatula, and the mucus was weighed. The cecal mucus was transferred into 5 ml of cold 0.9% (wt/vol) sodium chloride. One milliliter was removed and fixed for hybridization. Dilutions were made and plated on L-broth agar with 100 μ g of streptomycin sulfate per ml for determination of viable counts of *S. typhimurium*. All manipulations were completed as quickly as possible.

Preparation of histological sections for hybridization. After the colonized and control mice were sacrificed, the ileum, cecum, and the proximal part of the colon (1 cm) were removed from each mouse. The cecum was cut in two halves. One half was immediately transferred to a phosphate-buffered 25% (vol/vol) formalin solution together with the ileal and colonic sections. The other half was used for determination of CFU per gram of cecum. The cecum and colon were dehydrated and embedded in paraffin prior to preparation of 5- μ m-thick cross sections by use of a Spencer American Optical Rotation microtome 820. Sections were placed onto coated microscope slides (SuperFrost*Plus; Menzel-Gläser, Braunschweig, Germany) and kept at 4°C . Prior to hybridization, the sections were deparaffinated by treatment (three times; 10 min each) with xylene (Bie & Berntsen, Rødovre, Denmark) and dehydrated for 10 min in 96% ethanol. Before the hybridization solution was applied, the intestinal sections were circumscribed with a hydrophobic PAP-pen (Daido Sangyo Co. Ltd., Tokyo, Japan).

Oligonucleotide probes. A probe specific to *S. typhimurium* 16S rRNA (SAL998; 5'-TCTCTGGATTCTTCTGTGGA-3') was selected on the basis of the ribosomal sequences from 18 *Salmonella* species including *S. arizona*, *S. pullorum*, *S. typhimurium*, *S. typhi*, *S. enteritidis*, *S. phoenix*, *S. cholerae*, and *S. bongori*. The specificity of the probe was tested by use of the CHECK-PROBE program, The Ribosomal Database Project, University of Illinois (12). The probe was labelled at the 3' end with CY3 fluorescent dye (cyanine dye CY3.29-OSu; Biological Detection Systems, Pittsburg, Pa.). In addition, probe EU338 (25) which is specific to the eubacterial domain, was used. This probe was labelled with fluorescein (Peninsula Laboratories, Inc., Belmont, Calif.) at the 5' end.

Hybridization. Bacterial cell smears were hybridized on Teflon-coated slides as previously described (2, 21, 22). For visualization of bacteria in the intestinal sections, the eubacterial probe labelled with fluorescein was used in combination with the CY3-labelled, *Salmonella*-specific probe. For quantitative purposes, only SAL998-CY3 was used, and the hybridized cells were counterstained with 4',6'-diamidino-2'-phenylindol (DAPI) (Sigma).

Histological staining. After charge-coupled device (CCD) image capturing from the hybridized histological sections, the oil used for microscopy was removed by treating the slides with xylene and ethanol for 10 min, respectively. The slides were then stained with Alcian Blue and Mayers hematoxylin by the method of Nielsen et al. (19).

Microscopy and image analysis. Microscopy and image capturing were carried out essentially as previously described (22). Microscope filter sets 1 and 10 (Carl Zeiss) and XF40 (Omega Optical, Brattleboro, Vt.) were used to visualize DAPI, fluorescein, and CY3, respectively. The integration time for the CCD camera varied between 500 and 4,000 ms, depending on the intensity level of the hybridized bacteria. Long integration times were avoided for intestinal sections because of the very strong autofluorescence from the eucaryotic tissue. The same area containing the bacteria visualized by hybridization was found again after histologically staining the sections, and CCD pictures corresponding to the CCD pictures of the hybridized bacteria were captured through the 63x/1.25 Plan Neofluar Ph3 (phase-contrast) oil objective. Integration times for the CCD camera varied between 100 and 500 ms.

For quantification of ribosomes in bacterial cell smears, image analysis was done in 12 bits by using the PMIS software version 2.11 (Photometrics) and the Cellstat program (16). Hybridized cells were automatically circumscribed by use of Cellstat giving the cell size, fraction of dividing cells, and mean fluorescence intensity. In order not to bleach the lissamine CY3 prior to capturing images, cells were counterstained with DAPI, which was used for focusing the camera. At least three different images of 30 to 200 cells from each sample were quantified. The standard deviations of these measurements were 8 to 25%, giving standard errors of the means between 0.6 and 4.6%.

Measuring distances from bacteria to epithelium. From colonic histological sections prepared either 1 or 3 days after inoculation, CCD images captured through the XF40 filter set and thus showing only *Salmonella* bacteria and the autofluorescent epithelial cells were printed out as pictures (18 by 18 cm). For *S. typhimurium* SL5319 and SL5325, four and five pictures, respectively, were chosen because they showed areas where the mucosal layer had not been torn away from the epithelium during histological preparation. On these outprints, the shortest distance from each bacterium to the epithelial wall was measured and converted to the corresponding distance on the original section. For *S. typhimurium* SL5319, the fractions of bacteria observed in the mucosal layer that were associated with the epithelium on days 1 and 3 was determined.

Superimposing digitalized images. Captured CCD PMIS images were transformed to the TIFF format by using the PMIS software. TIFF images were colorized and superimposed by using Microsoft Adobe Photoshop version 3.0 or 2.5. Eucaryotic tissue appears red or orange because of autofluorescence.

Statistics. Standard errors of the means were obtained by division of the standard deviation on all measurements with the square root of the number of measurements. The mean intensity/volume values and average distances to the epithelium obtained for the two *Salmonella* strains were compared by the Student's *t* test.

RESULTS

Growth rates and ribosome contents of *S. typhimurium* SL5319 and SL5325 in defined laboratory media. Studies of pure *S. typhimurium* cultures were performed in minimal medium (AB) supplemented with amino acids and aromatic compounds necessary for growth of the two *Salmonella* strains. In addition, the minimal medium contained the following carbon sources (all percentages are wt/vol): (i) 0.4% glucose and 1% Casamino Acids, (ii) 0.4% glucose, (iii) 0.4% mannitol, or (iv) 0.5% glycerol. Minimal medium containing carbon sources i to iv supported generation times for *S. typhimurium* SL5319 (smooth) of 40, 64, 72, and 85 min, respectively. When cultures of *S. typhimurium* SL5325 (rough) were grown in minimal medium containing carbon sources i to iv, doubling times of 35,

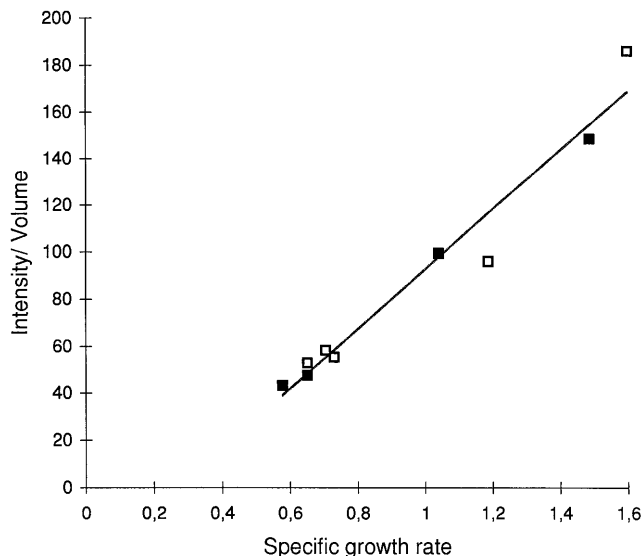


FIG. 1. Bacterial rRNA content inferred by whole-cell hybridization of balanced cultures of *S. typhimurium* SL5319 (■) and SL5325 (□) grown in defined laboratory media supporting different specific growth rates. The standard errors of the means are so small that they are covered by symbols.

57, 59, and 64 min, respectively, were measured. Growth in rich medium (L broth) supported generation times of 28 min for the smooth strain and 26 min for the rough strain of *S. typhimurium*. Samples of balanced cultures with the doubling times mentioned above were fixed and hybridized with a fluorescence-labelled, *Salmonella*-specific 16S rRNA probe, and after the digital images were captured, the light intensity per bacterial volume was determined. A linear correlation between the specific growth rate and the intensity per bacterial volume (i.e., the ribosome concentration) was observed (Fig. 1). Supporting this result, chemical determinations of the RNA and protein contents in cultures of *S. typhimurium* SL5319 and SL5325 showed that their RNA/protein ratios were the same, when growing at the same rate (data not shown).

Ribosomal contents of *S. typhimurium* SL5319 and SL5325 in the mouse cecum. To quantify the relative concentrations of ribosomes of *S. typhimurium* SL5319 and SL5325 growing in the mouse intestine, two mice were infected with about 2.2×10^8 CFU of each strain. The relatively low inoculum was chosen for this type of studies in order to be sure that the analyzed cells originated from proliferation in the gut and were not merely representing the inoculum. On day 6 after infection, the four mice were euthanized, and samples were taken for ribosomal hybridization and plate counts.

For both strains, the numbers of CFU per gram were found to be in the range of 10^8 to 10^9 in cecal mucus. For each mucosal fraction, the hybridization signal per bacterial volume was measured (see Materials and Methods for details), and by using the linear correlation between growth rate and ribosome concentration shown in Fig. 1, the hybridization signals were translated into apparent growth rates. For both strains of *S. typhimurium*, SL5319 and SL5325, the ribosome contents corresponded to generation times between 27 and 50 min in the cecal mucus. The average volume of the bacterial cells as determined by the Cellstat program (16) was observed to be the same in all fractions (data not shown).

Effect of crude mucus on the hybridization signal. In order to verify that binding to mucus or coating of the bacteria with a layer of mucus could not influence the hybridization signal,

the following experiment was performed. Pure cultures of *S. typhimurium* SL5319 and SL5325 were grown in minimal medium. Samples were taken, fixed, and hybridized. From the same cultures, other samples were concentrated and mixed with undiluted, nonsterilized mucus from mouse ceca in such a way that the bacterial density corresponded to the density observed in the cecum in vivo (i.e., 10^{10} bacteria per g of mucus). After incubation for 1 h at 37°C, bacterial smears were prepared, fixed, and hybridized in the same way as for in vivo smears from the intestine. The results showed the same intensity/volume ratio for the samples from minimal medium as for those that had been incubated in mucus, suggesting that in vitro binding of mucus to bacterial surfaces had no effect on the hybridization assay.

Spatial distribution of *S. typhimurium* SL5319 and SL5325, respectively, in mouse colon and cecum. To investigate the in vivo location in the mouse intestinal mucus of the two avirulent *Salmonella* strains, six mice were inoculated with 3.0×10^{10} CFU of either *S. typhimurium* SL5319 (smooth) or *S. typhimurium* SL5325 (rough). The high number of CFU in this inoculum was chosen in order to have a statistically significant number of bacteria on the CCD images showing cecal and colonic sections. On days 1, 3, and 6 after inoculation, two mice were euthanized. The total number of *S. typhimurium* per gram of cecum ranged from 10^{10} to 10^{11} in all cases. Histological sections of the cecum and colon were prepared and hybridized.

The total bacterial population in these sections was visualized by the fluorescein-labelled (green) eubacterial probe. The *S. typhimurium* cells were visualized by the specific CY3-labelled (red) probe. In the sections taken from the ceca of mice colonized with the avirulent *S. typhimurium* strains 1 day after infection, a large fraction of the smooth SL5319 bacteria, but only a few of the rough SL5325 cells were observed closely associated with the epithelium. On day 3 after infection, both of the *Salmonella* strains were present in the cecal mucosal layer but were rarely associated with the cecal epithelium (data not shown). The mucosal layer was frequently seen to be disrupted in cecal sections. This problem was overcome by looking at colonic sections, where the mucosal layer was found to be intact.

Figures 2 and 3 show the spatial distribution of strains SL5319 and SL5325, respectively, in the colon on days 1 and 3 after infection. On day 1, many of the smooth *S. typhimurium* SL5319 cells were associated with the colonic epithelial cells. The picture shown in Fig. 2A shows that also many of the indigenous bacteria (green) are associated with the epithelium together with the smooth *S. typhimurium* cells (red). However, in the cecal sections taken 1 day after infection, the smooth SL5319 *S. typhimurium* cells represent a large majority of the epithelium-bound cells (data not shown). On day 3, the smooth *Salmonella* cells were still distributed throughout the mucus, but only a few were bound to the epithelium (Fig. 2B). In the colonic sections from day 1, most of the LPS-deficient SL5325 bacteria were found in the part of the mucosal layer adjacent to the intestinal lumen (Fig. 3A). At 3 days after infection, they also appeared to be located in this part of the mucosa (Fig. 3B). Observations from pictures of intestinal sections made 6 days after inoculations were similar to the observations from day 3 (not shown).

By hybridization of histological sections of the ileum and jejunum of colonized mice, where CFU numbers for both of the two strains were found to be several orders of magnitude lower than those of the cecum and colon, only very few *S. typhimurium* cells were detected (13). In the uninfected control mice, no bacteria were observed to bind the oligonucleotide probe specific for *S. typhimurium*. In all sections from colo-

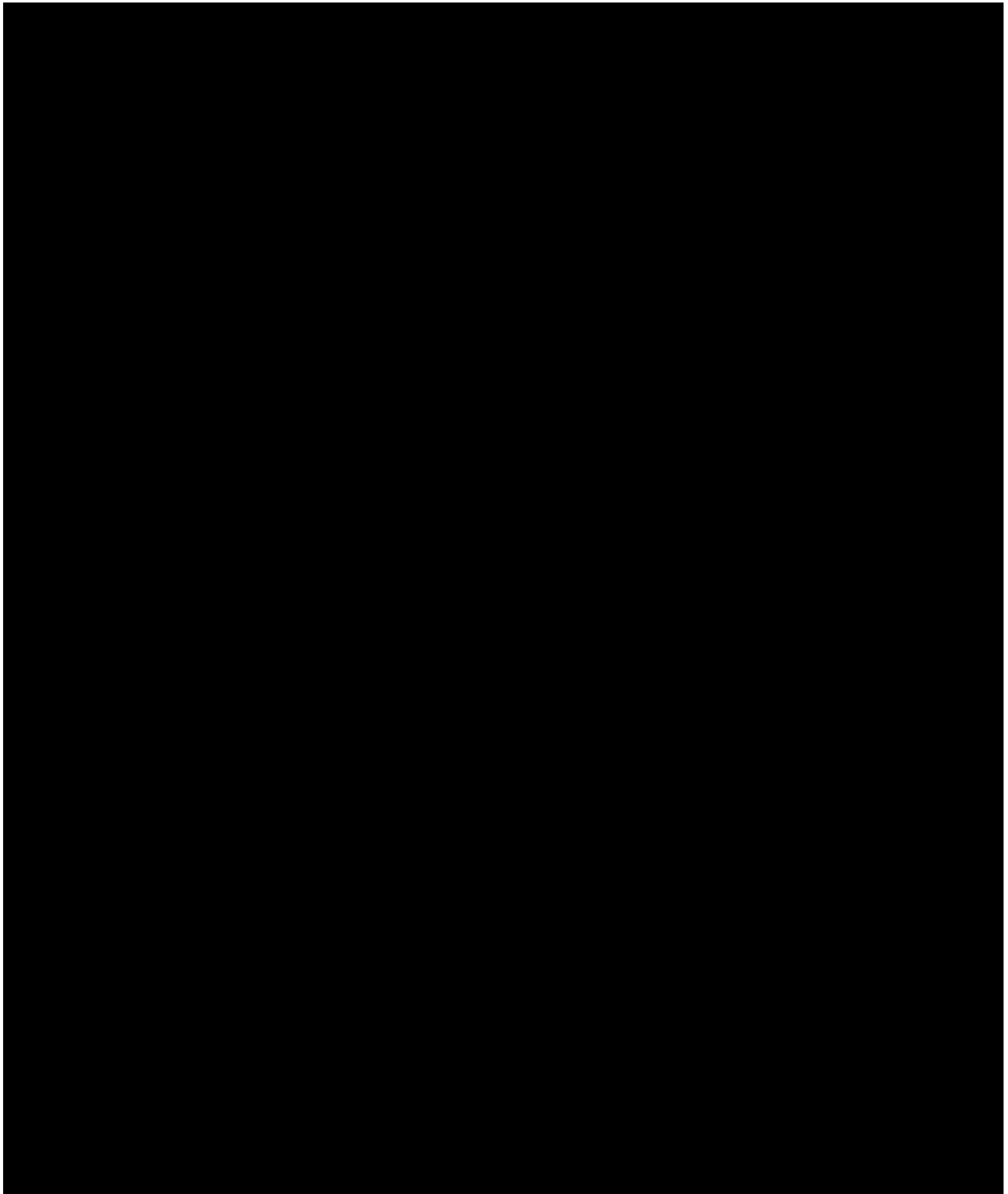


FIG. 2. Colonic sections of mice inoculated with *S. typhimurium* SL5319 (smooth) on day 1 (A) and day 3 (B) postfeeding. (Left) In situ hybridization with fluorescence-labelled oligonucleotide probes. *S. typhimurium* cells appear red, while all other eubacteria appear green. The colonic epithelium is labelled Ep and appears red or orange because of the strong autofluorescence of the eucaryotic tissue. (Right) Phase-contrast picture of the corresponding area stained with hematoxylin, which stains eucaryotic and procaryotic membranes, and with Alcian Blue, which stains the mucosal layer. Bars, 10 μ m.

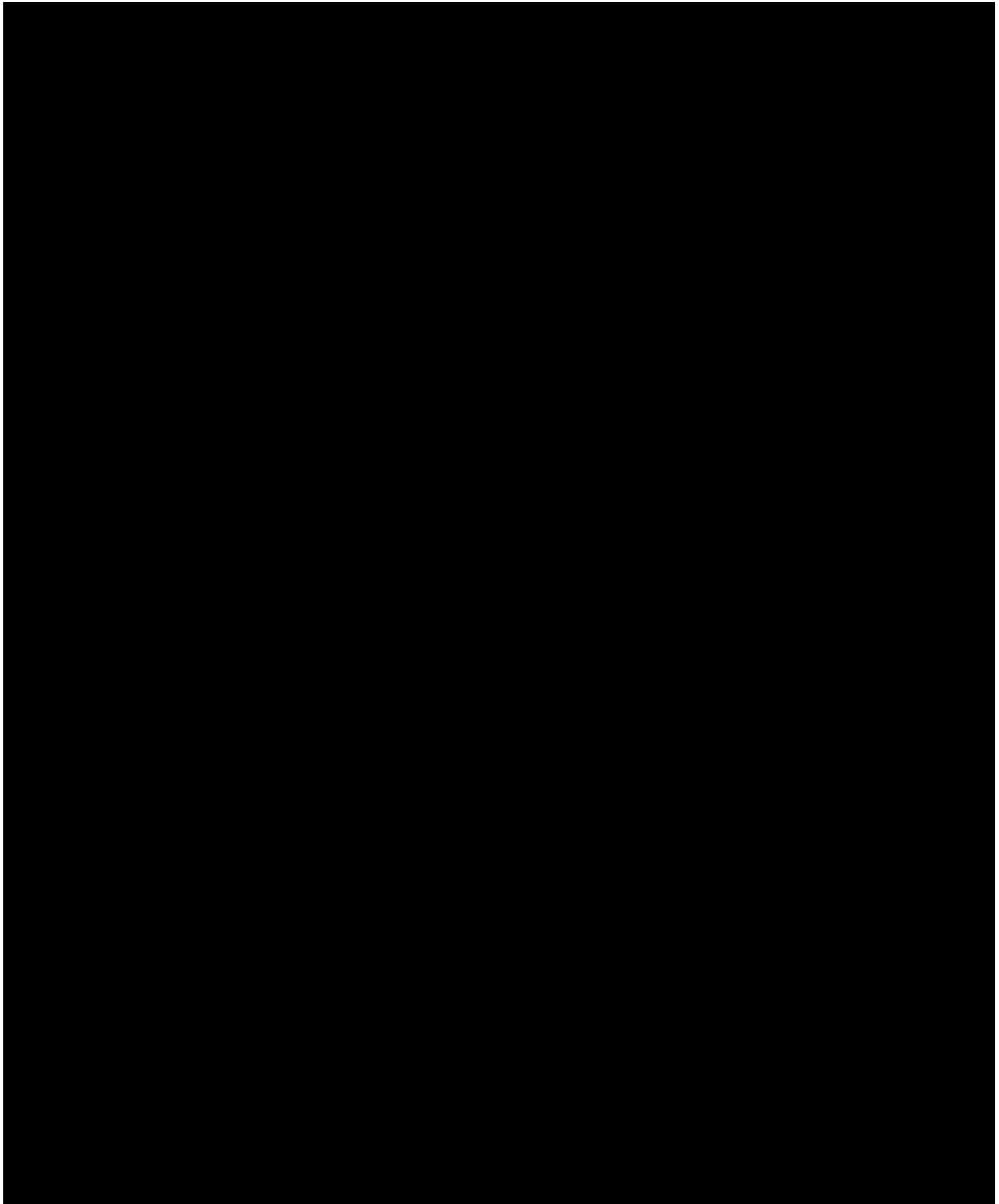


FIG. 3. Colonic sections of mice inoculated with *S. typhimurium* SL5325 (rough) on day 1 (A) and day 3 (B) postfeeding. (Left) In situ hybridization with fluorescence-labelled oligonucleotide probes. *S. typhimurium* cells appear red, while all other eubacteria appear green. The colonic epithelium is labelled Ep and appears red or orange because of the strong autofluorescence of the eucaryotic tissue. (Right) Phase-contrast picture of the corresponding area stained with hematoxylin, which stains eucaryotic and procaryotic membranes, and with Alcian Blue, which stains the mucosal layer. Bars, 10 μ m.

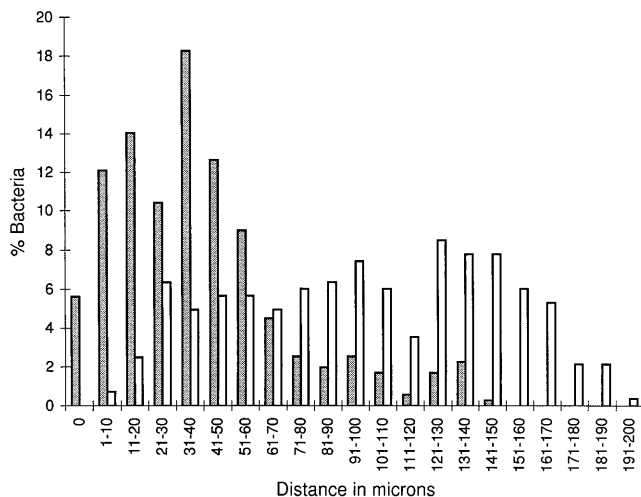


FIG. 4. Relative distributions of distances to the intestinal wall 1 and 3 days after inoculation of the smooth *S. typhimurium* SL5319 (stippled bars) or the rough *S. typhimurium* SL5325 (white bars) when fed separately to streptomycin-treated mice. The fraction of bacteria presented as having a distance of 0 μm are those that were directly associated with the epithelium. The average distance for the smooth bacteria was 40 μm , and the standard deviation on these measurements was 32 μm . The average distance for the rough bacteria was 100 μm , and the standard deviation was 47 μm . The higher standard deviation for the rough bacteria, which were mainly confined to the surface of the mucosal layer, can be explained by the variation of the thickness of the mucus.

nized as well as from noncolonized mice, the eucaryotic tissue appeared very autofluorescent at red, green, and blue wavelengths, whereas fluorescence from the probed bacteria could be seen only through the filter corresponding to the fluorochrome in question.

Quantitative determination of distances from *Salmonella* bacteria to epithelium. For each of the two *S. typhimurium* strains studied, four or five digitalized pictures of histological sections prepared 1 and 3 days after inoculation and showing areas from colon where the mucus was not disrupted and had not been torn away from the epithelium were chosen. On these pictures, the shortest distance from each *Salmonella* bacterium to the epithelium was measured. The results are shown in Fig. 4. The average distance to the epithelium measured for *S. typhimurium* SL5319 (smooth) was 40 μm , whereas the corresponding average for *S. typhimurium* SL5325 (rough) was 100 μm . The measurements of distance from bacterial cells to epithelium showed a larger standard variation for the rough strain than for the smooth strain. This could be explained by the observation, that most of the rough *S. typhimurium* cells were located at the surface of the mucosal layer, which is very variable in thickness. The two averages were compared by the Student's *t* test, showing that they are significantly different ($P \leq 0.005$). On day 1, the fraction of smooth *S. typhimurium* SL5319 in mucus, which were associated with the epithelium was 9.8%, whereas the corresponding fraction on day 3 was 2.1%.

DISCUSSION

It has previously been reported that the two avirulent, streptomycin-resistant *S. typhimurium* strains used in this study differ in their abilities to colonize the intestines of streptomycin-treated mice. A number of studies led to the suggestion that the ability of an *S. typhimurium* strain to colonize the intestine may depend partly on its ability to penetrate the mucosal layer and subsequently colonize the mucosa. This ability seemed to

be reduced for the rough *S. typhimurium* SL5325 strain compared with the smooth, but otherwise closely related, SL5319 strain. It was shown that none of these *S. typhimurium* strains grow in intestinal contents. Therefore, the present work has focused on localization and growth of the *Salmonella* strains in the intestinal mucosal layer (17, 18, 24).

The aim of this study was to investigate the colonization characteristics of *S. typhimurium* SL5319 and SL5325 by (i) quantitative and specific in situ rRNA hybridization on bacterial cells collected from colonized, streptomycin-treated mice in order to estimate in vivo ribosome concentrations and apparent growth rates in the intestinal mucosal layer and (ii) specific in situ rRNA hybridization on histological sections from the ceca and colons of these mice in order to visualize the spatial distribution of each of the two *S. typhimurium* strains in the mucus.

Quantitative in situ rRNA hybridization with fluorescent oligonucleotide probes targeting specific sequences on bacterial rRNA has previously been useful in estimation of apparent bacterial growth rates in the intestine (20, 22). In the present study, in situ hybridization was performed on a series of pure *S. typhimurium* cultures growing in laboratory media with generation times ranging from 26 to 85 min. The mean fluorescence intensity per bacterial volume (i.e., the mean ribosomal concentration) was quantified by digital image analysis. A linear correlation was observed between the specific growth rate and the fluorescence per bacterial volume (Fig. 1), as was earlier seen for other strains (20, 22). By using this correlation, the apparent growth rates of *S. typhimurium* SL5319 and SL5325 in the mouse intestinal mucus were estimated. On day 6 after infection, the mean fluorescence intensity per volume of bacterial cells taken from cecal mucus resulted in generation times between 27 and 50 min for both of the *S. typhimurium* strains. It should be noted that this is lower than the generation times previously estimated for *E. coli* BJ4 in the same environment (22).

In the present context, the important point is that the average ribosomal contents of the two *S. typhimurium* SL5319 and SL5325 appear to be the same, from which we conclude that they have the same potential for growth in the intestine when fed to mice separately.

The results of the specific ribosomal hybridizations performed on histological colonic sections of colonized mice support the hypothesis suggested by Nevola et al. (17) that the rough SL5325 strain gets trapped in the mucus and that it does not travel through the mucosal layer with the same ease as its smooth counterpart, strain SL5319. In the colonic sections, most of the LPS-deficient *S. typhimurium* cells were confined to the outer part of the mucus, while the bacteria carrying wild-type LPS were evenly distributed throughout the mucosal layer. Quantitative measurements of the distances from bacteria to the colonic epithelium showed averages of 40 and 100 μm for the smooth strain and the rough strain, respectively (Fig. 4). The standard deviations of the distances measured for the rough *S. typhimurium* SL5325 were larger than the corresponding standard deviation for the smooth *S. typhimurium* SL5319. This can be explained by the fact that the thickness of the colonic mucosal layer varies a lot and that the rough *Salmonella* cells were mainly confined to the surface of the mucus.

We do not mean to conclude from the similar ribosomal contents observed for *S. typhimurium* SL5319 and SL5325 when fed to mice separately that no growth rate competition is occurring when the two strains are fed to mice together. It has been shown (10) that two bacterial strains that are competing for the same nutrients but have different growth rates can coexist in the intestine in a stable manner only if the slowly

growing strain has specific adhesion sites available. We speculate that in a mixed-infection experiment as performed by Nevola et al. (17), the smooth *S. typhimurium* SL5319 strain is able to use up most of the nutrients released from the intestinal epithelium before they diffuse to the surface of the mucosal layer. This would result in a nutrient limitation of the rough *S. typhimurium*, which is confined to the mucosal surface. The rough *S. typhimurium* would, therefore, in a mixed-infection experiment have a slower growth rate than its smooth counterpart and thus be washed out of the intestine.

Another finding of this study was that the two *S. typhimurium* strains differ markedly in their binding to the host epithelium *in vivo*. In the hybridized cecal and colonic cross-sections, almost no SL5325 cells were found associated with the epithelial cells either on day 1 after infection or later in the colonization phase (Fig. 3 and 4). However, many of the smooth SL5319 cells found in mucus were binding to the cecal and colonic epithelium on the first day after infection (Fig. 2 and 4). It is unlikely that this difference in binding, which we have observed in many independent cases, was due to a higher binding affinity of the smooth strain, since it has previously been shown *in vitro* that the efficiency of binding to MDCK epithelial cell lines (3, 9) was an order of magnitude higher for SL5325 (rough) than for SL5319 (smooth) (4). Furthermore, it was seen that the LPS-deficient mutant bound to preparations of mouse epithelial cells at levels three to five times higher than the wild-type LPS strain (17). Therefore, it seems highly unlikely that the LPS-deficient *S. typhimurium* cells would not bind to the epithelium *in vivo*, if they were able to get in contact with the intestinal wall. On the basis of these observations, we suggest that the difference in binding to the epithelium *in vivo* observed for the two strains is caused by the reduced ability of the rough strain to penetrate the mucosal layer as discussed above.

Quantitative determinations of the fractions of smooth *S. typhimurium* cells in the mucosal layer that were associated with the colonic epithelial wall showed that 9.8% of the observed bacteria were bound to the intestinal epithelium 1 day after oral infection, whereas this fraction was reduced to 2.1% on day 3. We speculate that the avirulent *S. typhimurium* strain might induce a host immune response preventing the bacteria from subsequent binding to the epithelial cells, but not from proliferating in mucus. Recent reports show that commensal enteric bacteria can in fact induce a specific secretory immunoglobulin A response upon colonization of the gut (24) and that secretory immunoglobulin A alone can prevent infection of invasive bacteria including *S. typhimurium*, presumably by immune exclusion at the mucosal surface (15, 26). We are currently designing experiments to test whether the avirulent *S. typhimurium* strains used in this study cause such a response.

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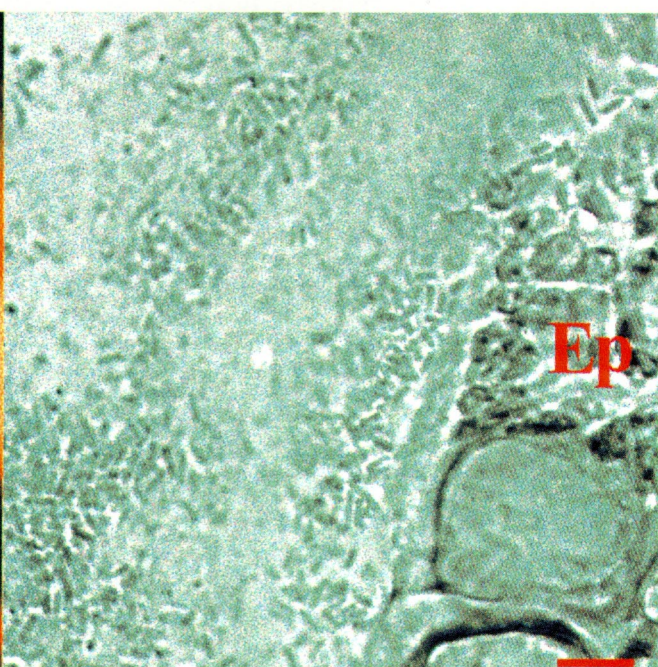
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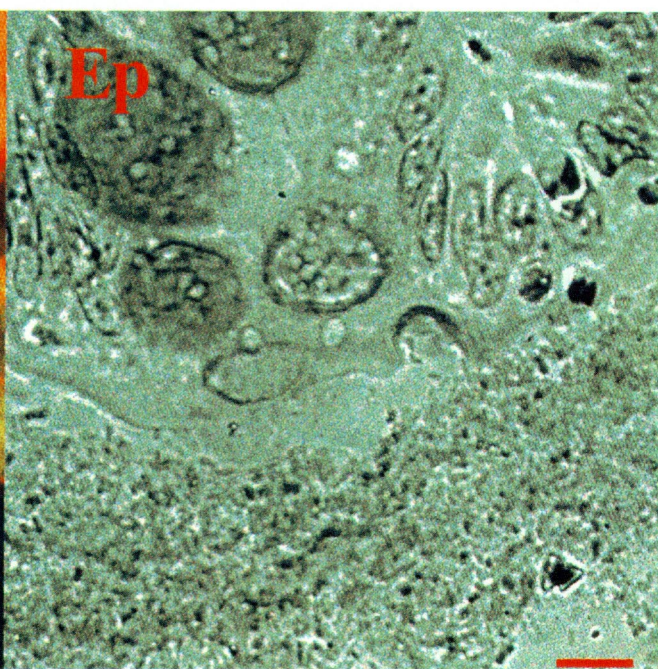
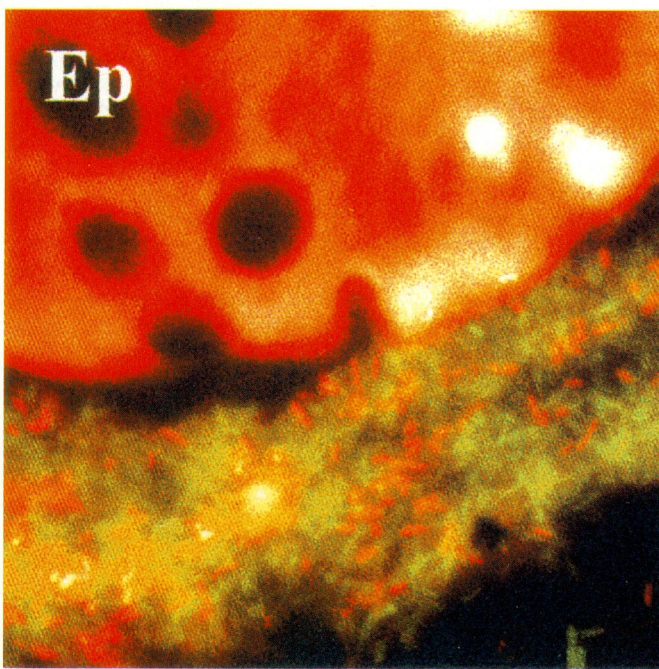
REFERENCES

- Al-Ramadi, B. K., J. M. Greene, J. J. Meissler, Jr., and T. K. Eisenstein. 1992. Immunosuppression induced by attenuated *Salmonella*: effect of LPS responsiveness on development of suppression. *Microb. Pathog.* **12**:267-278.

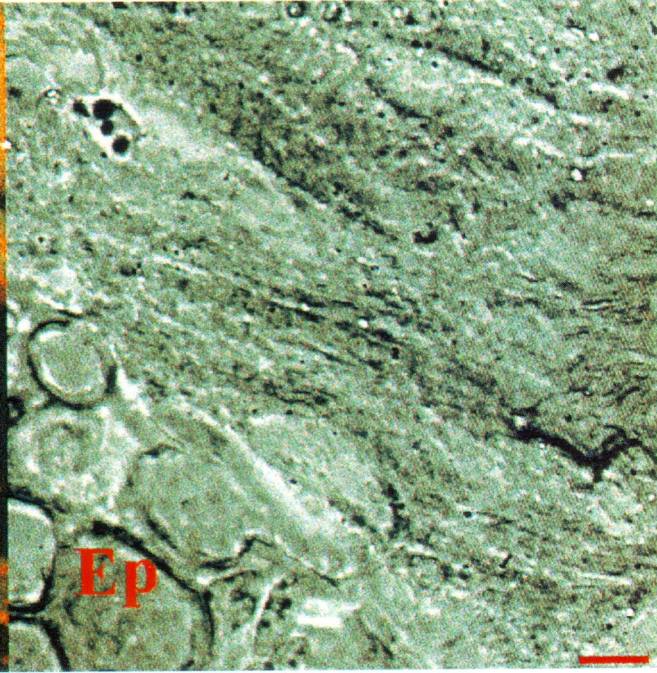
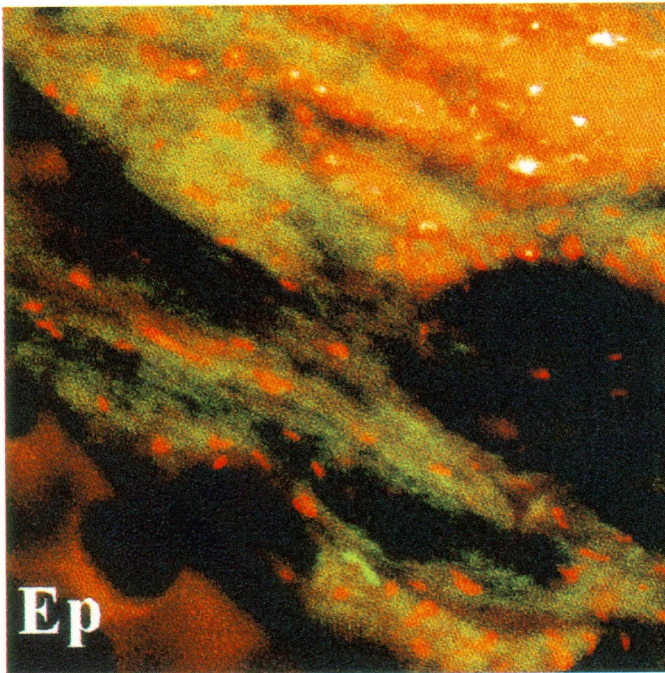
- Amann, R. I., L. Krumholz, and D. A. Stahl. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* **172**:762-770.
- Betts, J., and B. B. Finlay. 1992. The identification of *Salmonella typhimurium* invasiveness loci. *Can. J. Microbiol.* **38**:852-857.
- Brown, D. Unpublished data.
- Camprubi, S., S. Merino, J. F. Guillot, and J. M. Tomás. 1993. The role of the O-antigen lipopolysaccharide on the colonization *in vivo* of the germfree chicken gut by *Klebsiella pneumoniae*. *Microb. Pathog.* **14**:433-440.
- Clark, D. J., and O. Maaløe. 1967. DNA replication and the division cycle in *E. coli*. *J. Mol. Biol.* **23**:99-112.
- Cohen, P. S., J. C. Arruda, T. J. Williams, and D. C. Laux. 1985. Adhesion of a human fecal *Escherichia coli* strain to mouse colonic mucus. *Infect. Immun.* **48**:139-145.
- Ding, H. F., I. Nakoneczna, and H. S. Hsu. 1990. Protective immunity induced in mice by detoxified *Salmonella* lipopolysaccharide. *J. Med. Microbiol.* **31**:95-102.
- Finlay, B. B., B. Gumbiner, and S. Falkow. 1988. Penetration of *Salmonella* through a polarized Madin-Darby canine kidney epithelial cell monolayer. *J. Cell Biol.* **107**:221-230.
- Freter, R., H. Brickner, J. Fekete, M. M. Vickerman, and K. E. Carey. 1983. Survival and implementation of *Escherichia coli* in the intestinal tract. *Infect. Immun.* **39**:686-703.
- Käthy, H. 1985. Antibody response to bacterial surface components. *FEMS Symp.* **25**:96-97.
- Larsen, N., G. J. Olsen, B. L. Maidak, M. J. McCaughey, R. Overbeek, T. J. Macke, T. L. Marsh, and C. R. Woese. 1993. The ribosomal RNA database project. *Nucleic Acids Res.* **21**:3021-3023.
- Licht, T. R., L. K. Poulsen, S. Molin, and K. Krogfelt. Applications of ribosomal *in situ* hybridization for the study of bacterial cells in the mouse intestine. *In Proceedings of the Academy Colloquium of the Koninklijke Akademie van Wetenschappen. Ecology of pathogenic bacteria: molecular and evolutionary aspects, in press.*
- McCormick, B. A., B. A. D. Stocker, D. C. Laux, and P. S. Cohen. 1988. Roles of motility, chemotaxis, and penetration through and growth in intestinal mucus in the ability of an avirulent strain of *Salmonella typhimurium* to colonize the large intestine of streptomycin-treated mice. *Infect. Immun.* **56**:2209-2217.
- Michetti, P., M. J. Mahan, J. M. Schlauch, J. J. Mekalanos, and M. R. Neutra. 1992. Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen *Salmonella typhimurium*. *Infect. Immun.* **60**:1786-1792.
- Møller, S., C. S. Kristensen, L. K. Poulsen, J. M. Carstensen, and S. Molin. 1995. Bacterial growth on surfaces: automated image analysis for quantification of growth rate-related parameters. *Appl. Environ. Microbiol.* **61**:741-748.
- Nevola, J. J., D. C. Laux, and P. S. Cohen. 1987. *In vivo* colonization of the mouse large intestine and *in vitro* penetration of intestinal mucus by an avirulent smooth strain of *Salmonella typhimurium* and its lipopolysaccharide-deficient mutant. *Infect. Immun.* **55**:2884-2890.
- Nevola, J. J., B. A. D. Stocker, D. C. Laux, and P. S. Cohen. 1985. Colonization of the mouse intestine by an avirulent *Salmonella typhimurium* strain and its lipopolysaccharide-defective mutants. *Infect. Immun.* **50**:152-159.
- Nielsen, E. M., J. Schlundt, A. Gunvig, and B. L. Jacobsen. 1994. Epithelial, mucus and lumen subpopulations of *Escherichia coli* in the large intestine of conventional and gnotobiotic rats. *Microb. Ecol. Health Dis.* **7**:263-273.
- Poulsen, L. K., G. Ballard, and D. A. Stahl. 1993. Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. *Appl. Environ. Microbiol.* **59**:1354-1360.
- Poulsen, L. K., F. Lan, C. S. Kristensen, P. Hobolth, S. Molin, and K. A. Krogfelt. 1994. Spatial distribution of *Escherichia coli* in the mouse large intestine inferred from rRNA *in situ* hybridization. *Infect. Immun.* **62**:5191-5194.
- Poulsen, L. K., T. R. Licht, C. Rang, K. A. Krogfelt, and S. Molin. 1995. Physiological state of *Escherichia coli* B14 growing in the large intestines of streptomycin-treated mice. *J. Bacteriol.* **177**:5840-5845.
- Schneider, W. C. 1945. Phosphorus compounds in animal tissues. Extraction and estimation of desoxyribose nucleic acid and of ribose nucleic acid. *J. Biol. Chem.* **161**:293-303.
- Shroff, K. E., K. Meslin, and J. J. Cebra. 1995. Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut. *Infect. Immun.* **63**:3904-3913.
- Stahl, D. A., and R. I. Amann. 1991. Development and application of nucleic acid probes, p. 205-248. *In E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics.* John Wiley and Sons, New York.
- Steinmetz, I., P. Schiffmann, and B. Brenneke. 1995. Secretory monoclonal IgA class-switch variants against bacterial enteric pathogens in bile and intestinal secretions. *FEMS Immunol. Med. Microbiol.* **11**:329-336.



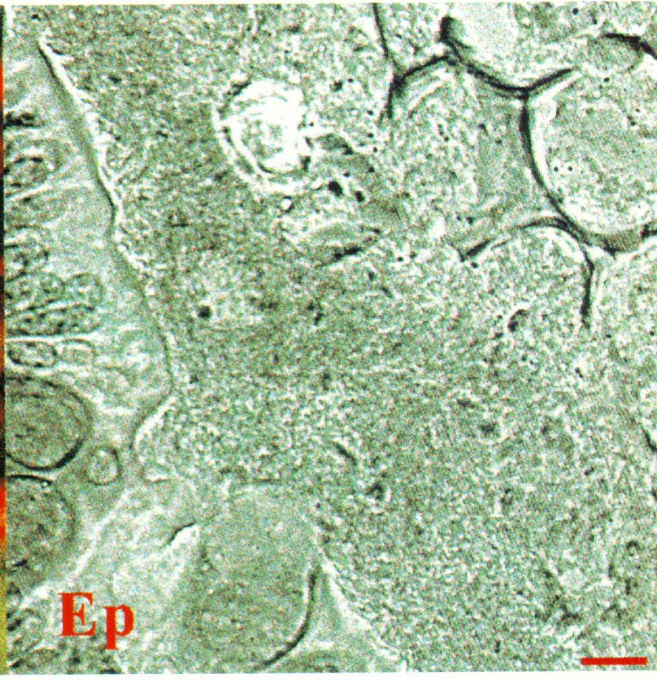
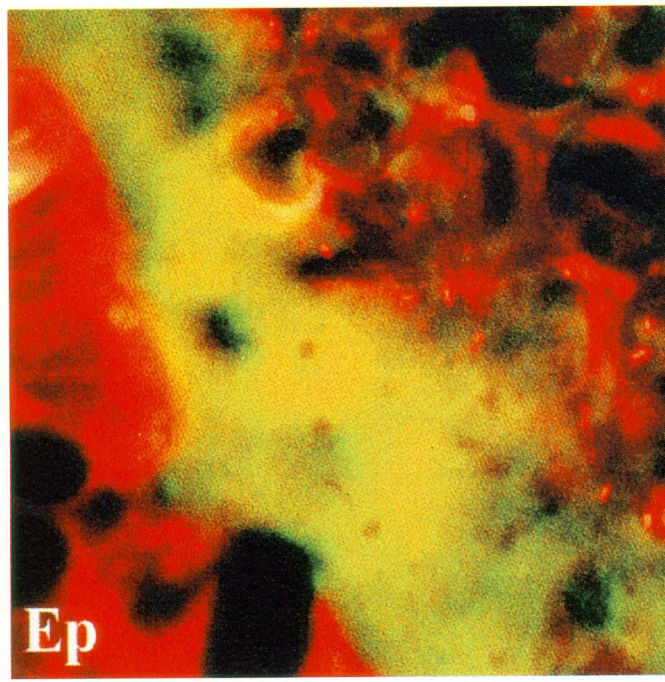
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